REDUCTION OF *ENTEROCOCCUS FAECALIS* BIOFILM BY BLUE LIGHT AND SODIUM HYPOCHLORITE

by

Daryl A. Kwan

Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, 2017.

Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

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Richard L. Gregory

_____

Ygal Ehrlich

_____

Masatoshi Ando

_____

Susan Zunt

_____

Josef S. Bringas

_____

Kenneth J. Spolnik
Chair of the Research Committee
and Program Director

Date _____

ACKNOWLEDGMENTS

There's a saying, "The days are long, but the years are short." I look back to the first day I visited the program to meet with Dr. Spolnik and the residents at the time, and now we are quickly approaching the end of the program.

I'd first like to thank my family for its support, sacrifice, and encouragement. To my parents, who always pushed me to be better and saw potential in me: The two of you did whatever it took to allow me to succeed, and I can never repay you.

My dearest wife, Katie: We have had too many good times to count since freshman year of college. Thank you for the never-ending patience and happiness that you bring to my life. You helped to provide me with the ability to go back to residency and chase my dreams. I love you a lot.

To our commander-in-chief, Dr. Spolnik. Not only are you the clinician I can only aspire to be, but you are also one of the calmest and tactful people I know. You seem to have most, if not all, the answers when something comes up. Your leadership and decision-making has made our endodontic program one of the strongest in the nation. Thank you for accepting me into the Indiana University family. I feel truly blessed to have trained under you, and I will need to keep your number on speed dial.

Dr. Bringas, thank you for everything. Your clinical advice and guidance has been invaluable over the past two years. You push us to excel past our limits while still being practical. The program would not be the same without you, and we feel the impact of your absence even when you go to Manila for a week or two. I can't wait to

spend 8 more months with you talking about food, cars, and most importantly, endodontics.

Dr. Warner and Dr. Ehrlich, thank you for all your help and guidance over the past two years. Having full-time faculty who are passionate about what they do provide for a more enjoyable experience.

This thesis would not have come together without Dr. Gregory. Thank you for everything you contribute to the field of research. It's remarkable how much you accomplish at IUSD with your teaching on top of all the student projects you have going on at once. Even more amazing is that you stay on top of everything and give 100 percent to each individual project. You are a great advisor, and I am lucky to have worked with you.

To all part-time faculty: Drs. Adams, Berman, Deardorf, Hine, Hill, Sahni, Steffel and Vail; one of the greatest strengths of our program is the knowledge that you bring week after week. Thank you for taking the time to come and give us your input. Being able to see different clinical techniques from a wide array of providers has been extremely beneficial to the development of our clinical skills.

Diane, thank you for your ceaseless dedication to the program. We are not the most organized group, so I'm not sure how we'd survive without you telling us where to be and what to do.

Thank you to all assistants for keeping things flowing and the schedule on track. I know there were stretches when we were down one and things got hectic in the clinic, but all of you picked up the slack to make it through. I will cherish the time and conversations we had in the operatory.

Special thanks to Dr. Ando for being on the research committee and for your instrumental help in this project.

One of the things I will miss greatly is the time spent with my co-residents in the conference room. We've shared many laughs and random discussions about too many purely hypothetical situations. The camaraderie that Indiana's program provides is second to none and I am very glad to have met and known all of you. I hope our friendship continues beyond graduation and that we all stay in touch while showing each other our successes and failures.

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INTRODUCTION

The clinical outcome for an endodontically treated tooth is dependent on the elimination of microbes from the canal system. However, complete disinfection of the canal space can be difficult, if not impossible, due to the complex root anatomy (1-3). Parts of the canal wall remain untouched even with thorough preparation (4,5), which often leaves contaminated dentin in the canal space (6,7). Currently, disinfection of a tooth involves mechanical debridement (8) with the use of chemical irrigation using sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) and chlorhexidine. An interappointment medicament such as calcium hydroxide ($Ca(OH)_2$) can also be used to enhance disinfection and reduce the microbial load (9). The most widely used irrigant in endodontics is NaOCl. It has the ability to penetrate tubular dentin to approximately 130 μm in depth (10). However, dentinal tubules can be infected at up to 1,000 μm (11) which would mean that even with with high concentrations of NaOCl, microbes could continue to evade complete disinfection if they had penetrated deep within these dentinal tubules.

NaOCl used in higher concentrations can cause cytotoxicity as evidenced by an in vitro study which showed that the viability of human dental pulp stem cells increased as the concentration of NaOCl decreased (12). This is of particular importance in regenerative endodontic procedures (REP) where current guidelines suggest using a low concentration of 1.5-percent NaOCl for disinfection. Success of regenerative therapy requires stem cells from the apical papilla, a scaffold and growth factors which are derived from platelets or the dentin itself (13,14). Most importantly, however, REP is dependent on the disinfection of the tooth. Therefore, one of the goals of REP should be

to maximize disinfection while using the lowest concentration of NaOCl possible in order to limit cytotoxicity to apical stem cells.

There have recently been new advancements for disinfection of the root canal system using photodynamic light therapy (PDT) (15,16). Conventionally, PDT uses a nontoxic photosensitizer (PS) and a light source (17). Light can activate and excite the photosensitizer, which reacts with molecular oxygen. The result is a highly reactive oxygen species (ROS) which can go on to destroy and kill microorganisms (18,19).

There has been research at the Indiana University School of Dentistry investigating PDT, particularly the antibacterial effect of blue light on *Streptococcus mutans*. Results from these studies indicate that visible violet-blue light of the electromagnetic spectrum has the ability to inhibit *S. mutans* growth and reduce the formation of *S. mutans* biofilm without the use of an exogenous photosensitizer (20). It is suspected that there are endogenous porphyrins in bacteria that can be excited using a light source, thereby releasing ROS (21,22).

For the purposes of this study, a monospecies biofilm consisting of *Enterococcus faecalis* was used. *E. faecalis* was chosen because numerous studies have shown that it is highly associated with persistent, secondary infections of failing root canals, even more so than primary endodontic infections (23-32). It is a facultatively anaerobic, gram-positive coccus (33) with multiple virulence factors such as aggregation substance, surface adhesins, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin (34) which makes the bacteria difficult to kill and remove. However, the most significant reason for resistance is its microbial arrangement in a biofilm.

In the mid 1960s, apical periodontitis was found to be related to microbial infection and microscopic techniques showed bacteria in the infected root canal space organized as a biofilm (35). More recently, histopathological studies have been used to assess and confirm that apical periodontitis is indeed a microbial biofilm based process (36). It is also known that bacterial biofilm is much more resistant to removal than planktonic bacteria (37).

Therefore, we chose to grow a monospecies biofilm of *E. faecalis* so we could investigate how it responded to blue light with varying concentrations of NaOCl and if there were any synergistic effects when these two disinfection techniques were used together. These findings may suggest that we may be able to use reduced concentrations of NaOCl to disinfect teeth when used in conjunction with PDT.

Clinical Significance

If blue light enhances the effect of NaOCl then a lower concentration of NaOCl may be used and this may allow for a more thorough disinfection of canal walls. This would be of benefit for regenerative endodontics in which low concentrations of NaOCl are recommended due to cytoxocity to apical stem cells by NaOCl. It is expected that the light will make the biofilm more susceptible to disruption by low concentrations of sodium hypochlorite. We expect that the *E. faecalis* biofilm will be easier to disrupt after the exposure to blue light versus that of our no light groups.

OBJECTIVE

- Specific Aims: To investigate the effectiveness of blue light at 380 nm to 440 nm to reduce adherence of *E. faecalis* biofilm followed by NaOCl irrigation at various concentrations.

HYPOTHESES

- Null: Blue light and NaOCl will not have an effect against *E. faecalis* biofilm adherence.
- Alternative: Blue light when used in conjunction with NaOCl will have a synergistic effect in reducing adherence of *E. faecalis* biofilm

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Some of the most ancient findings related to toothaches come from Sumerian texts dating back to 5000 BC that describe "tooth worms" as the cause of caries (38). It was believed that the cause of toothaches would be tooth worms that would wiggle around inside a tooth after they bore holes. The pain stopped once the worm had stopped wiggling. There is also evidence that the Chinese used acupuncture around 2700 BC to treat pain from decayed teeth. In the Beers Papyrus written by the Egyptians between 1700 BC and 1500 BC with material dating as far back as 3700 BC, light was shed on medical practices used to treat the "throbbing of Benut blisters in teeth" (39).

Since these ancient practices, there have been many texts showing society's attempt to relieve dental pain, maintain exposed pulp, and preserve teeth. The greatest advancement in the field of endodontics came from Pierre Fauchard, who is considered the father of modern dentistry. In his textbook, "Le Chirurgien Dentiste," he precisely described the dental pulp and was the first to dispel the myth of the "tooth worm" (40). In 1746 Fauchard described the removal of pulp tissue, but it was not until almost a century later that Edwin Maynard introduced the first root canal instrument, created out of a watch spring.

During the end of the 19th century, there were several important advancements in endodontics, such as the use of gutta percha cones as obturating material (41), the use of electric current to test pulp vitality (42), and the introduction of the rubber dam (43). However, it was not until 1895 that the most important contribution to medicine was created when Wilhelm von Roentgen accidentally discovered a new form of energy with

an unknown nature that had the ability to penetrate solid material, fittingly calling these rays of energy "X" rays (44). A few weeks later, a dentist from Brunswick, Germany, named Otto Walkhoff took the first dental radiograph.

Shortly after 1910, local anesthetics that were safe and effective were introduced and radiographic machines were getting better. It could be suspected that after this time, great strides would be made in the field of dentistry and endodontics. However, several years before this in 1904, Frank Billings called attention to the relation between oral sepsis and bacterial endocarditis. A few years later, one of Billings' students, E.C. Rosenow, introduced the theory of "focal infection" in a study investigating bacteria's role on root canal therapy. He showed that streptococci were present in many diseased organs and that they could be present in those organs from vascular spread from a distant site (45). He proposed that streptococci and other organisms can relocate from an apical granuloma to cause diseases at peripheral organs.

In the same year, Mayrhofer was able to link pulpal infections with specific microorganisms. He was able to show that streptococci were involved in roughly 96 percent of the cases studied. Also in that year, an English physician and pathologist named William Hunter lectured at the University of Montreal and spoke about sepsis and antisepsis in medicine. Hunter was very critical of dentistry and claimed that gold crowns were a "mausoleum of gold over a mass of sepsis" causing focal infections. He linked a wide area of systemic diseases such as gastritis, colitis, purpura, and "nervous disturbances of all kind" to the sepsis that was produced by teeth (46). It was therefore believed that systemic disease could be resolved by the removal of teeth.

For the next 40 or so years, dentists proceeded to extract any devitalized teeth, along with teeth that were vital with large restorations, inlays, crowns, or bridges. This was partly due to the fear of the effects of focal infections and partly because dentists did not want to perform the meticulous and difficult root canal therapy.

Thankfully, during the 1930s and 1940s, the idea of focal infections seemed to lose ground as more information came to light. For example, in 1937 Logan distinguished the difference between infection and presence of bacteria (137). In the same year, Burket showed 200 cases of arthritis that were unresolved even with removal of infective foci, indicating that although they were associated, infective foci did not cause arthritis (138).

Members of a small group of dentists including Coolidge, Johnson, Reihn, Callahan, Grove, Prinz, and others, worked to improve current endodontic procedures by "using aseptic techniques, bacteriological and histological methods, and X-rays for diagnostic purposes"(47). It is likely because of their efforts that the preservation of the pulpless tooth has survived. In addition, it wasn't until around the late 1940s or early 1950s that evidence and research showed that a devitalized tooth did not have a role in systemic disease (48).

In the 1940s, as root canal treatment became more common, a group of 20 dentists came together in Chicago to create an organization that would set the standard for endodontic treatment. This meeting resulted in the formation of the American Association of Endodontics (AAE) (49). In 1963 the ADA recognized endodontics as it own specialty and by 2015 the AAE had 8,015 members.

THEORY OF ENDODONTICS

Endodontic disease first begins when bacterial microorganisms are introduced into the root canal system (RCS). These microorganisms can enter the RCS by ways of trauma, a previous restoration, or caries. The bacterial insult on the pulp can lead to inflammation of the pulp tissue, eventually causing pulpal necrosis. The first study to show that pulpal and endodontic problems are related to microbial contamination of the RCS came in 1965 with the study by Kakehashi, Stanley and Fitzgerald (50). In this study, the researchers observed pathologic changes resulting from untreated experimental pulp exposures in germ-free rats as compared with conventional rats with complex microflora. Their data showed that, with the conventional rats, complete pulpal necrosis occurred with granulomas and abscess formation and evidence of repair was uniformly lacking. However, in the germ-free rats, no devitalized pulp, apical granulomas, or abscesses were found. This showed that bacteria were a significant factor in determining outcomes of healing.

Control of endodontic infection is a joint effort by several host and treatment factors (51). Success in all aspects of this cooperation will best guarantee elimination of infection and healing of any apical pathology. The necessary factors to control are host defense system, systemic antibiotic therapy (only when indicated), instrumentation and irrigation, locally used intracanal medicaments between appointments, root canal filling, and adequate coronal restoration. In addition to these elements, infection control must be maintained throughout the treatment process (52). From these elements, endodontic treatment can be broken down into three distinct phases: instrumentation, irrigation and

three-dimensional obturation of the RCS. Grossman highlighted 13 principles of effective root canal treatment (107):

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irritating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

The goal of instrumentation is to remove all the necrotic or vital organic tissue and give the canal system a shape that allows easy debridement. Following proper instrumentation, irrigants are used to enhance bacterial elimination and to ensure an aseptic environment. The last phase of therapy is to seal the RCS coronally and apically. The ability to properly obturate the RCS is primarily dependent on the quality of the canal instrumentation and the clinical skill of the clinician.

INSTRUMENTATION

The goal of instrumentation and irrigation is to remove and/or kill all the microorganisms in the RCS, and to neutralize any antigenic/biological potential of the microbial components left in the canal (53). The principles of root canal preparation are to remove all organic debris and microorganisms from the RCS, and to shape the walls of the root canal to facilitate the cleaning and subsequent obturation of the entire root canal space. In some cases, all the treatment can be finished in one appointment; however, in cases where it is not feasible, the goal of instrumentation and irrigation is to create an optimal condition for the placement of an antibacterial intracanal medicament to enhance the disinfection of the canal. The process of mechanical instrumentation is the core method to reduce the bacterial load in an infected root canal. A study done by Bynstrom and Sundqvist (54) showed that with instrumentation alone, a reduction of bacterial numbers would be 100-to 1000-fold, but achieving a bacteria-free root canal system with mechanical instrumentation alone is difficult. A tooth rarely has a single simple root canal. Many root canal systems can have accessory canals, lateral canals, fins, anastomoses between canals, or an apical delta, and the majority of these features are not entirely accessible to conventional instrumentation (55).

IRRIGATION

The use of irrigation solutions is an important part of effective chemomechanical preparation of the tooth. Irrigation facilitates removal of necrotic tissue and residual bacteria from accessory canals or bacteria that was not effectively removed during instrumentation. In addition, irrigants can prevent the extrusion of hard or soft tissue into the periapical area (56). The most commonly used irrigant is NaOCl. At a pH of 11,

NaOCl primarily exists as HClO, which is considered to be the active moiety responsible for bacterial inactivation by NaOCl (57). HClO has been found to disrupt oxidative phosphorylation and other membrane associated activities (58). It has also been suggested that DNA synthesis can be affected and NaOCl is best known for its strong bactericidal activity. NaOCl is used in concentrations ranging from 0.5 percent to 5.25 percent. Decreasing the concentration of the solution reduces its toxicity, antibacterial effect, and ability to dissolve tissues, and increasing the concentration or warming the solution increases its effectiveness (59). There are major disadvantages to the use of NaOCl as an irrigant. These include its cytotoxicity when extruded out the apex into the periradicular tissue, its foul smell and taste, and its ability to corrode metal objects (60). In addition, NaOCl does not kill all bacteria encountered in endodontic infection, does not have any substantivity, nor does it remove the smear layer.

In order to overcome NaOCl's cytotoxicity issues, chlorhexidine (CHX) was introduced as an endodontic irrigant. CHX gluconate has been used for many years for caries prevention, in periodontal therapy, and as an antiseptic mouthwash (61). The reason CHX might be used over NaOCl is because it has a lower level of cytotoxicity to stem cells, lacks a foul smell and taste, and has good substantivity for up to 12 weeks. However, unlike NaOCl, it cannot dissolve organic substances and necrotic pulpal tissue; and like NaOCl, chlorhexidine is unable to remove the smear layer or kill all bacteria (62). CHX is cationic and exerts its antimicrobial effect by electrostatically binding to bacteria and disrupting the cell wall (63,64). However, there is concern with use of CHX due to its ability to form a harmful precipitate when mixed with NaOCl. Initially, it was thought that this precipitate was a toxin called Para-chloroaniline (PCA) (65). However,

further studies have determined that the precipitate was para-chlorophenylurea (PCU), which is a parachlorophenylguanidyl-1, 6-diguanidyl-hexane (PCGH) (66). Regardless, one should avoid precipitation formation by using another irrigant in between using these two solutions (67).

Due to NaOCl's limitations with removal of the smear layer, EDTA, and other chelating agents such as tetracycline and citric acid are used for removal of the organic portion of the smear layer. Without removal of the smear layer, NaOCl cannot penetrate the dentinal tubules and has a limited effect (68). NaOCl is still used as an adjunct solution for removing the remaining organic components. The common method recommended is irrigation with 17-percent EDTA for one minute followed by a final rinse with NaOCl to remove the smear layer (69). EDTA has little to no antibacterial effect (70), but studies have shown that removing the smear layer improves the sealing ability of the root canal filling material (71).

OBTURATION

The obturation phase of endodontic treatment is to prevent the reinfection of the root canals that were biomechanically cleaned, shaped and disinfected by instrumentation, irrigation, and medication procedures. Successful obturation is accomplished with the use of materials and techniques capable of densely filling the entire RCS (72). In addition, it is important to avoid any gross overextension or underfilling in the presence of a patent canal (73). Studies have concluded that the success rate for an obturated tooth is much higher when the material ends 0 mm to1 mm from the radiographic apex (74).

Microorganisms and their byproducts are the major causes of periapical disease; however, it is difficult to totally disinfect the root canal system, so using using three-dimensional obturation provides an impermeable seal within the entire RCS. Controversy has surrounded root canal obturation for many years, and clinicians and researchers have compared many warm obturation techniques without finding a proven superior. This allows clinicians to experiment and form their own preferences through trial.

MICROORGANISMS

Endodontic infection is classified as primary or secondary. The classification relies on if the tooth has been previously treated. There are different types of bacteria that are associated with primary and secondary infections that are typically organized in a biofilm. The term biofilm is used to describe a thin-layered collection of microbes that can form communities that increase their chances of survival (108).

Primary endodontic infections are typically comprised of gram-negative, anaerobic rods (109,110). In 2014 Nagata demonstrated that infected immature teeth had a similar microbial composition to that of primary endodontic infections and averaged 2.13 species in the RCS (111). The most commonly found species in immature teeth is *Actinomyces naeslundii*, a facultative, anaerobic, gram-positive rod (111). *A. naeslundii* has also been found in primary endodontic infections and its pathogenicity is related to the activation of the innate immune system of the host, which triggers cytokines and the inflammatory process (112-115).

Another bacterium found in primary endodontic infections is *Fusobacterium nucleatum*, a gram-negative rod. *F. nucleatum* is a critical component of biofilm

formation because it can attach to numerous different gram-positive and negative bacteria, serving as a middle colonizer. It is able to create a host immune response by invading host tissue cells (116).

Porphyromonas gingivalis, a gram-negative obligate anaerobe can also be found in approximately 50 percent of primary endodontic infections. Its main virulence factors include LPS, lipoproteins, capsule, and fimbrae on top of many others (116-118). It is considered a black-pigmented species typically unable to survive prolonged exposure to NaOCl (119).

Secondary endodontic infections are primarily composed of gram-positive facultative cocci. There is an average of 1.3 species in secondary endodontic infections, according to Nair (110,120). The most common bacterium associated with persistent, secondary endodontic infections is *E. faecalis* which is a gram-positive, facultative anaerobe also present in primary endodontic infections (121). *E. faecalis* has many virulence factors including lipoteichoic acid, aggregation substance, cytolysis, lytic enzymes, and pheromones (122). Furthermore, it can invade deep into the dentinal tubules thereby avoiding calcium hydroxide and resisting its high pH (108,117,120,123). For these reasons, *E. faecalis* biofilm is challenging to remove and to eradicate from the canal space, especially in immature permanent teeth with open apices.

IMMATURE TEETH WITH PULPAL NECROSIS

Follow up studies have concluded that conventional endodontic therapy has a 97-percent success rate (75); however, the prognosis is not as good for immature teeth with pulpal necrosis. The pulp is integral to continue apexogenesis of the young permanent tooth. It is important to not disrupt this process because long-term retention of the

permanent tooth requires a root with a favorable crown to root ratio and dentinal walls that are thick enough to withstand normal function. Therefore, the primary goal of treatment of young permanent dentition is to preserve vitality if possible (76). If vitality cannot be maintained, obturation of these teeth can be challenging due to the higher likelihood of the filling material through the open apex of the immature tooth (77). There has been an evolution of treatment options for these teeth over the past few decades. Two methods of treating immature permanent teeth with pulpal necrosis are through apexification or regenerative therapy.

APEXIFICATION

Apexification is a method of inducing root end closure of an incompletely formed vital permanent tooth by removing just the coronal, non-vital radicular tissue just short of the root end. A biocompatible agent is then placed for a period of two to four weeks in order to disinfect the canal space (78). $Ca(OH)_2$, with its high pH can denature microbial proteins and induces a mineralized apical barrier by causing a low-grade irritation inside the canal space. Apexification requires good patient compliance due to the prolonged waiting period for the apical barrier to form (79). Depending on the stage of root development, complete formation of the calcified apical barrier may be prolonged and require multiple sessions to achieve this goal (80).

In 1995 mineral trioxide aggregate (MTA) was introduced as a root-end filling material (81). MTA, similarly to $Ca(OH)_2$, possesses a high pH and creates an apical hard tissue barrier, more consistently than $Ca(OH)_2$ (82). Due to MTA's hydrophilic properties, it is able to form a better apical seal in the presence of moisture than $Ca(OH)_2$ (83). Instead of needing multiple appointments as with $Ca(OH)_2$, MTA serves as an

apical stop, so that obturation can be completed in the same visit. This is more convenient for the patient and clinician and makes compliance less of an issue. One study reported an 81-percent success rate of MTA apexification at 1 year (84) and another reported 93.5-percent success rate at 1.5 years (85). Even with the improvement in apexifcation techniques, long-term prognosis for immature teeth with pulpal necrosis is still compromised due to thin walls and short roots, leaving these teeth prone to fracture. Recently, regenerative endodontics has emerged as a solution to this problem, leading to better prognosis for immature teeth with pulpal necrosis.

REGENERATIVE ENDODONTIC PROCEDURES (REP)

Successful REP requires four main elements: stem cells, scaffolds, and growth factors and disinfection (87). It is based on the use of biologically based procedures to regenerate missing or damaged tissue such as dentin, root structures and cells of the pulp-dentin complex (88). What distinguishes regenerative healing from repair is the nature of the replaced tissue. True regeneration is only when the new tissue formed is identical to the replaced tissue in structure and function. On the other hand, repair through healing occurs when the new tissue has a loss of structure or function or is not identical to the original tissue type (89).

HISTORY OF REP

Multiple attempts have been made at pulpal regeneration, but the first attempt was reported in literature by Nygaard-Ostby in 1961 and further reestablished in 1971 (90,91). The article described a procedure where pulp tissue was removed from vital teeth and bleeding was induced within the canal followed by partial root canal filling. They

reported that during histological investigation, connective tissue formation was identified. However, the reason their experiment ultimately failed was due to crown-down leakage (91). Meyers and Foundain in 1974 infected the pulps of teeth in monkeys. The root canals were biomechanically cleaned and instrumented 2 mm beyond the apex and the teeth were disinfected with 5.25-percent NaOCl; the apical constrictions of the mature teeth were enlarged, and bleeding was induced into the canals. Very limited in-growth of tissue was observed at the apical extent.The authors suggested that much of this could be due to periapical inflammation and colonies of microorganisms found in the canals of the majority of the cases. It is worth noting though that immature teeth responded better than the mature teeth; they demonstrated continued root growth and the largest amount of connective tissue in-growth (92).

In 1976 Nevins removed dental pulps of rhesus monkeys after exposing the RCS to oral contaminants for one week in an attempt to create an endodontic infection. The teeth were temporized with cotton pellet and IRM and three days later, a collagen phosphate gel was placed into the canals. After 12 weeks, histological observation showed “revitalization” of the canals with differing types of soft and hard connective tissue including “cementum, bone, and reparative dentin” (93).

Bose et al. found that in their study, there was greater root growth in both width and length after REP than in apexification alone (94). In 2012 Jeeruphan et. al. also performed a study which cited a 28.2-percent increase in width and 14.9-percent increase in length after REP was performed, which was significantly more than that of MTA or $Ca(OH)_2$ apexification (95). In 2014 Kahler et al. published a case series that found resolution of the periapical pathology in 90.3 percent and complete apical closure in 19.4

percent at 1.5 years of 16 consecutive regenerative procedures (96). These studies suggest that REP has a significant ability to promote root development.

INDICATIONS AND OUTCOME FOR REP

REP has mostly been used on immature teeth with open apices due to research that has shown that apical diameters greater than 1 mm are more likely to undergo revascularization (97). The definition of success for REP needs to be established in order to properly assess the outcomes of treatment. For this reason, the AAE outlined three goals to measure the success of regenerative endodontics (98):

1) Resolution of symptoms and apical/radicular healing.

2) Continued root development and formation.

3) Positive response to pulp sensibility testing.

DISINFECTION FOR REP

Studies by Kakehashi et al., indicated that healing occurs only in germ-free rats (50), and Thibodeau et al. (99) demonstrated that vital tissue only formed when teeth were disinfected during REP, shedding light on the importance of proper disinfection strategy for regenerative success. Commonly, the disinfection strategy of choice for REP is a combination of NaOCl followed by an intracanal medication like $Ca(OH)_2$ or an antibiotic paste including TAP (100).

NaOCl has several desirable characteristics including excellent bactericidal efficacy (101), tissue dissolution capacity, and effective lubrication for endodontic instruments (102); however, it has several disadvantages when performing REP. First, NaOCl has been found to have detrimental effects on stem cells of the apical papilla in

higher concentrations (103,104). In addition, dentin conditioning with NaOCl at its maximum clinically used concentration leads to greatly diminished odontoblast-like differentiation (105). Furthermore, the elastic modulus and flexural strength of dentin is decreased when NaOCl is used at 3.0-percent and 5.0-percent concentrations. Therefore, current guidelines recommend the use of 1.5-percent concentration of NaOCl during REP disinfection at the first appointment and to avoid use of NaOCl at the second appointment while stem cells are being induced into the canal space via blood (49).

The most recent recommendations for regenerative endodontic procedures from the AAE as of June 2016 are (106):

Case Selection

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.
- Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

Informed Consent

- Two (or more) appointments.
- Use of antimicrobial(s).
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable).

- Permission to enter information into AAE database (optional).

First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20-ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised (1.5-percent NaOCl, 20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals with paper points.
- Place $Ca(OH)_2$ or a low concentration of TAP. If the TAP is used: 1) Consider sealing pulp chamber with a dentin bonding agent (to minimize risk of staining) and 2) Mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/ml.
- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3 mm to 4 mm of a temporary restorative material such as Cavit™, IRM™, glass ionomer or another temporary material. Dismiss patient for 1 week to 4 weeks.

Second Appointment (1 week to 4 weeks after first visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3.0-percent mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 ml of 17-percent EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (Induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cement-enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix (AFM).
- Stop bleeding at a level that allows for 3 mm to 4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, CollaCote™, CollaTape™ or other material over the blood clot if necessary and use white MTA as capping material.
- A 3-mm to 4-mm layer of glass ionomer (e.g., Fuji IILC™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA should be considered in teeth where there is an esthetic concern.

- Anterior and premolar teeth – Consider use of CollaTape/CollaPlug and restoring with 3 mm of RMGI followed by bonding a filled composite to the beveled enamel margin.
- Molar teeth or teeth with PFM crown – Consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI or alloy.

Follow-up

- Clinical and Radiographic exam
- No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
- Resolution of apical radiolucency (often observed 6 mos to 12 mos after treatment)
- Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12 mos to 24 mos after treatment).
- Increased root length.
- Positive pulp vitality test response
- The degree of success of regenerative endodontic procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
- Primary goal: The elimination of symptoms and the evidence of bony healing.
- Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)

- Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

Due to the detrimental effects NaOCl can have on apical stem cells at higher concentrations, it is in the patient's best interest to use the lowest concentration of NaOCl possible when treating teeth for regenerative endodontics. Various disinfection protocols have been studied including the use of PDT.

PHOTODYNAMIC LIGHT THERAPY (PDT)

There have been many studies demonstrating the ability of blue light at specific wavelengths having a substantial antibacterial effect. Methicillin-resistant *Staphylococcus aureus* (MRSA) and community-acquired *S. aureus* (CRSA) have been killed using a high intensity narrow spectrum (HINS) wavelength at 405 ± 5 nm and 470 nm, respectively (124-126). In addition, using blue light has also shown susceptibility in *Propionibacterium acnes* at a wavelength of 407 nm to 420 nm (127,128), *Helicobacter pylori* at 405 nm (129), and *Pseudomonas aeruginosa* at 470 nm (130).

Early studies from the 1990s used PDT and photosensitizers to help enhance the killing of oral microbes. Some of these studies focused on *S. mutans*. The results indicated that *S. mutans* biofilm was more vulnerable with the use of certain photosensitizers like rose Bengal and erythrosine at 455 nm (131), only erythrosine at 500 nm to 500 nm, and using 450 nm with the use of another photosensitizer, curcumin (132). In addition, 99.5 percent of *S. mutans* were killed in the presence of toluidine blue with the use of blue light at 636 nm (133). It has also been shown that a combination of hydrogen peroxide and blue light at 400 nm to 500 nm can have an affect on *S. mutans* biofilm (134). Chebath-Taub et al. proposed the concept of delayed antibacterial activity

using photodynamic therapy by showing that the ability of *S. mutans* to form new biofilm is disrupted when the bacteria are subjected to blue light at 400 nm to 500 nm (135).

Previous research has demonstrated that PDT without the use of any exogenous photosensitizers has been used to destroy *Prevetolla melaninogenica*, *P. gingivalis*, *Prevetolla nigrescens*, and *Prevotella intermedia*. These oral black-pigmented periodontal bacteria are thought to contain endogenous porphyrins that are excited with exposure to blue light at 380 nm to 520 nm (136). Also, research at IUSD with Gomez et al. has demonstrated that blue light of the electromagnetic spectrum has the ability to inhibit *S. mutans* growth and reduce the formation of *S. mutans* biofilm without the use of an exogenous photosensitizer (20).

Therefore, it may be safe to assume that in the context of PDT, exogenous photosensitizers are catalysts that drive the killing of microbes when exposed to light. However, one may suspect that using a specific wavelength of light by itself has a key role in the photo inactivation process possibly affecting adherence and weakening the biofilm structure.

MATERIALS AND METHODS

BACTERIAL STRAIN AND GROWTH MEDIA

Anaerobic blood agar plates (CDC, BioMerieux, Durham, NC) were used to initially grow and maintain *E. faecalis* ATCC 29212. Tryptic soy broth (TSB, Acumedia, Baltimore, MA) supplemented with 1.0-percent sucrose (TSBS) was used to grow the bacterium at 37°C in a 5.0-percent CO2 incubator. Biofilms were grown in sterile 96 well flat bottom polystyrene microtiter plates (Fisher Scientific, Co., Newark, DE) using TSB supplemented with 1.0-percent sucrose (TSBS). One hundred ninety μl of TSBS was added to each well and inoculated with 10 μl of an overnight culture of *E. faecalis*. Biofilm cells were grown in 4 wells per group, and the distance between the biofilm wells prepared was kept apart at 1 well distance to reduce chances of light scatter. The plates were incubated for 72 hours at 37°C in a 5.0-percent CO_2 incubator. The reason 72-hour biofilm incubation was chosen instead of a three-week biofilm was because there was a limited amount of surface area in the microtiter well, which reduces the amount of bacteria that can grow compared with a dentin sample. Also, the chance of contamination to the biofilm was increased if the biofilm was grown for a greater amount of time.

Light Source

Quantitative light induced fluorescence (QLFTM/CLIN Inspektor Research System BV, Amsterdam, Netherlands), was used in this study. This technology typically uses fluorescence to detect early decay in teeth. The source of light was generated from a control unit with a 35 Watt Xenon arc lamp with a 5-mm external light source diameter. In order to extract blue light, the use of an optical high pass band filter was employed. A liquid-filled light guide was used to pass the light through. A laboratory grade

spectrometer (Model USB2000, Ocean Optics Inc., Dunedin, FL) was used to measure the intensity output. The setup of the spectrometer implemented the use of a fiber optic line that was connected to an integrating sphere linked to the spectrometer. The spectrometer was hooked to a computer that used SpectraSuite software (Ocean Optics Inc.) to analyze the light. Before the experiment was conducted, calibration of the equipment was performed using a National Institute of Standards and Technology (NIST) traceable light source (LS-1-CAL, Ocean Optics Inc.). Biofilm at the bottom of a single well of a 96-well microtiter plate was irradiated for 5 minutes from a distance of 2 cm from the light source. A stand was created for the light in order to standardize the 2-cm distance. The distance from the bottom of the well to the top was approximately 1.75 mm. Furthermore, the lens cover on the light source was 0.25 mm in height. In order to measure the intensity of the light at the top surface of the biofilm, the radiant power (mW) of the light was measured from the integrating sphere at a distance of 2 cm and that was divided by the area of the opening of the integrating sphere. Average intensity of the light was measured to be 30.872 mW/cm2 while the wells were being irradiated for 5 minutes of irradiation. The wavelength used was measured at between 380 nm to 440 nm with the peak wavelength at 405 nm. In the wavelength that was detected, there was a spectral overlap of violet and blue light; however, the term “blue light” was suggested by the manufacturer and was used in this study.

MICROTITER PLATE BIOFILM ASSAY

A biofilm assay was used to determine the effects of blue light on *E. faecalis* biofilm. The distance between the light source tip and the biofilm was maintained at 2 cm. Prior to exposure, the supernatant liquid was removed by pipetting and 5 continuous minutes of blue light was directly exposed to the wet biofilm. After light exposure, the biofilm was treated with 200 μL of 0.125%, 0.25%, 0.5% or 1% sodium hypochlorite for 30 seconds. Two hundred μL of 10-percent formaldehyde was added to fix the biofilm cells for 30 minutes. The biofilm cells were carefully washed twice and 200 μL of 0.5-percent crystal violet was added for a period of 30 minutes to stain the biofilm. The stained cells were washed three times and 200 μL of 2-propanol was added to extract the dye from the biofilm cells for 1 hour. The extracted biofilm cell dye was measured using a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA) at 490 nm, which provides quantitative information of the relative density of the biofilm cells exposed to blue light and with no blue light.

EXPERIMENTAL GROUPS

The various concentrations of sodium hypochlorite were 0.125%, 0.25%, 0.5% and 1%. Two 96-well microtiter plates were randomly assigned into 8 experimental groups (n = 4 per group). The groups consisted of only blue light, only NaOCl, and a combination of blue light with the varying concentrations of NaOCl. The experiment was repeated three times for a total of 12 samples per group.

Controls

Controls included biofilms of *E. faecalis* without blue light or sodium hypochlorite; *E. faecalis* with blue light and without sodium hypochlorite; *E. faecalis* without blue light and with sodium hypochlorite; a positive control with 0.12-percent CHX; and a sterility control with only media.

STATISTICAL ANALYSIS

The effects of exposure to blue light and NaOCl concentration were analyzed using 2-way ANOVA with factors for the two main effects and the interaction. Pair-wise comparisons were made using the Sidak method to control the overall significance level at 5.0 percent. A logarithmic transformation of the data will likely be necessary to satisfy the ANOVA assumptions. A p-value of 0.05 or less was considered to be statistically significant.

SAMPLE SIZE

Based on previous studies, the coefficient of variation was expected to be 0.34. With a sample size of 12 per blue light-NaOCl concentration combination, the study had an 80-percent power to detect a 50-percent difference with blue light versus without blue light, assuming two-sided tests were conducted at an overall 5.0-percent significance level.

RESULTS

A two-way ANOVA was conducted to compare the effects of 5 concentrations of NaOCl (0%, 0.125%, 0.25%, 0.5%, 1.0%), with and without blue light, and their interaction, on *E. faecalis* biofilm growth. Due to non-normality, a rank transformation was performed prior to analysis.

Decreasing the concentration of the NaOCl in the no-light- and blue-light-treated groups minimized the reduction of *E. faecalis* biofilm adherence thereby causing an increase in the absorbance of the crystal violet released from the biofilms cells (Figure 11 and Figure 12).

Overall, there was a significant effect for NaOCl alone and a significant effect for blue light alone on the reduction of *E. faecalis* biofilm adherence. Their combined interaction was also significant until the remaining bacterial biofilm and absorbance approached 0 (Figure 13). There was significantly more biofilm growth for treatment groups that used lower concentrations of NaOCl. Also, there was significantly more biofilm growth in the absence of blue light.

FIGURES AND TABLES

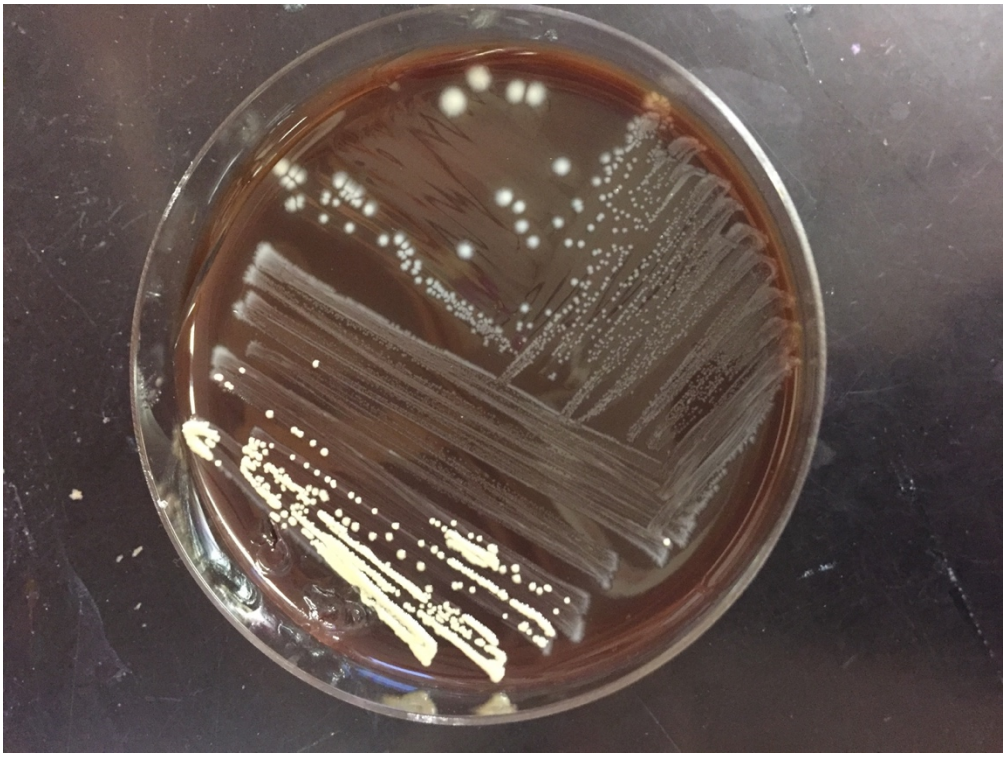

FIGURE 1. Blood agar plate containing *E. faecalis* after 24 h of incubation.

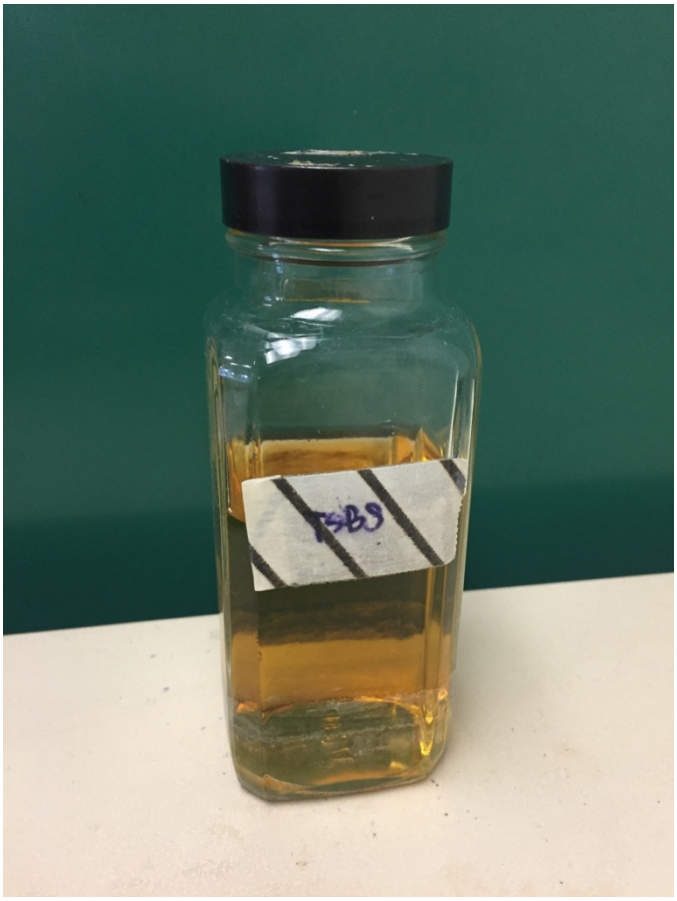

FIGURE 2. A sterile broth of tryptic soy broth (TSB) supplemented with sucrose (TSBS) was used to supply the clinical species with growth media.

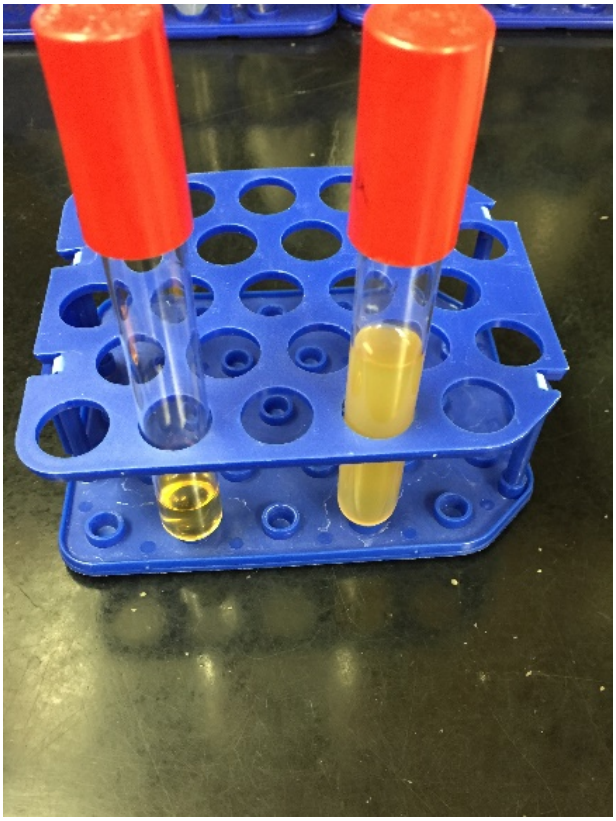

FIGURE 3. Sterile media on the left and on the right is a sample of 24 hour culture of *E. faecalis*. The cloudiness is indicative of bacterial growth.

FIGURE 4. Incubation of 96-well microtiter plates for 72 hours.

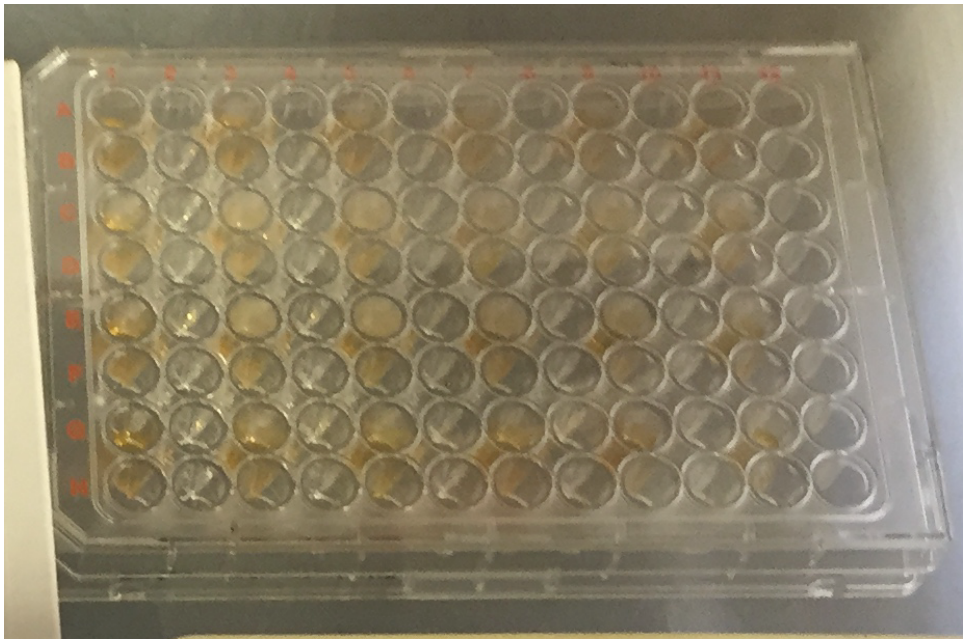

FIGURE 5. Microtiter plate wells after 72 hour of incubation and prior to being exposed to blue light.

FIGURE 6. Quantitative light-induced fluorescence (QLF), which was used to provide blue light.

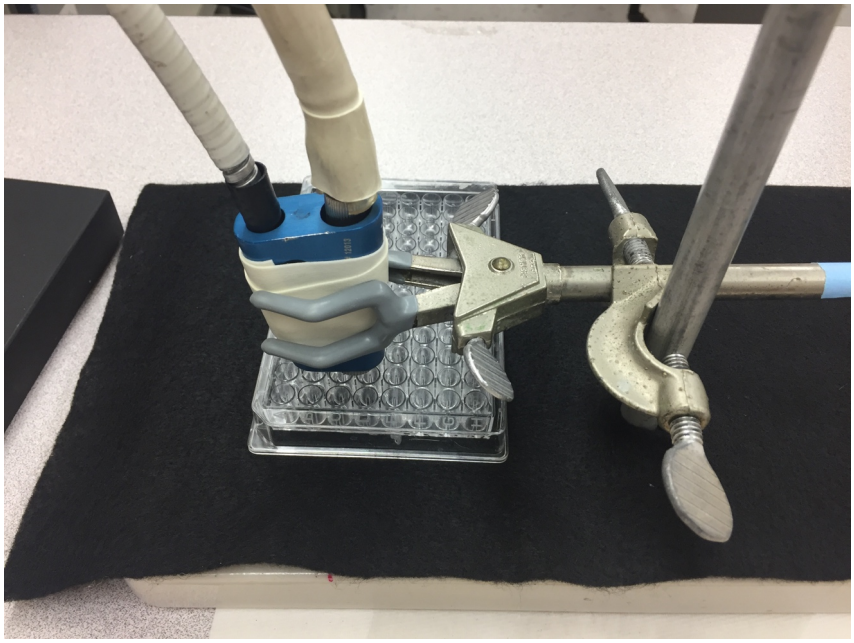

FIGURE 7. Close-up of the stand that was used to hold the light source at a 2-cm premeasured distance from the bottom of the microtiter plate wells.

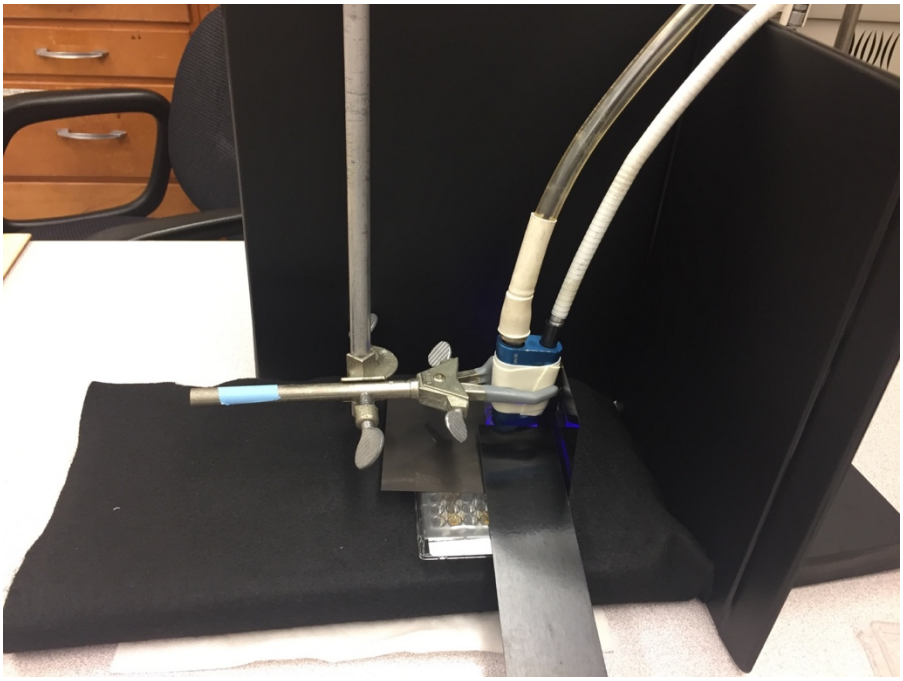

FIGURE 8. Experimental setup while QLF light was activated. Barriers were placed around and over wells to reduce light scatter.

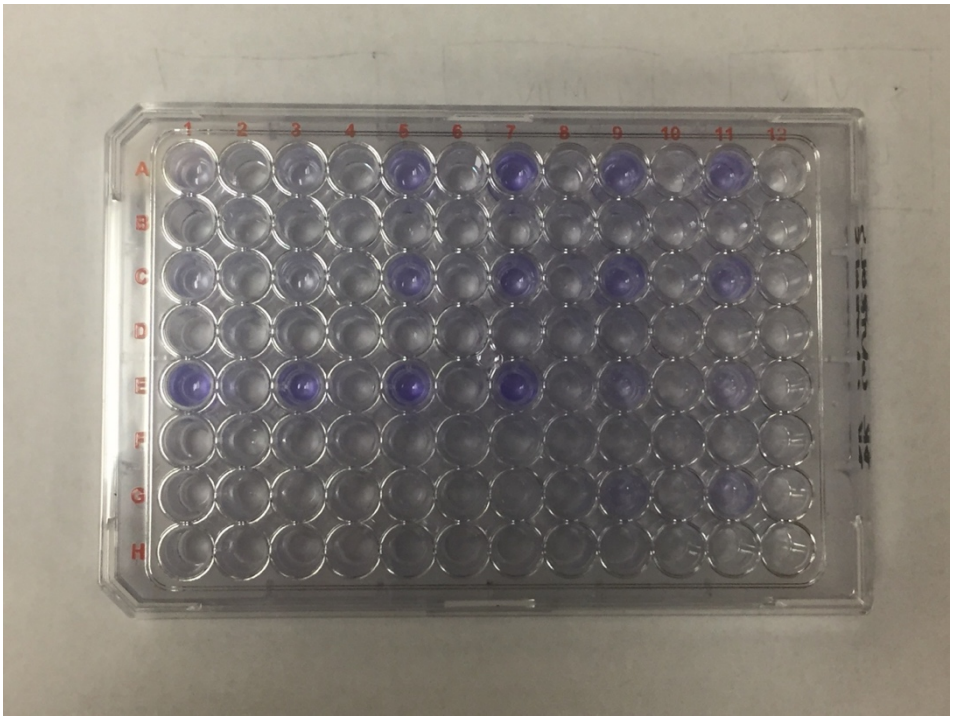

FIGURE 9. Note the varying degrees of the color of the wells after crystal violet staining.

FIGURE 10. The microtiter plate spectrophotometer that was used to obtain absorbance data.

TABLE I

Absorbance results from the spectrophotometer for groups treated with blue light and NaOCl

Light	NaOCl Concentration (%)	N	Mean	SD	SE	Median	IQR		Min	Max
No	0	12	0.103	0.014	0.004	0.101	0.094	0.108	0.082	0.127
No	0.125	12	0.073	0.011	0.003	0.0705	0.063	0.082	0.06	0.093
No	0.25	12	0.039	0.025	0.007	0.036	0.0185	0.062	0.003	0.079
No	0.5	12	0.029	0.052	0.015	0.015	0.002	0.03	-0.007	0.184
No	1	12	0.003	0.008	0.002	0.0045	-0.004	0.0085	-0.01	0.014
Yes	0	12	0.071	0.033	0.009	0.052	0.047	0.102	0.041	0.126
Yes	0.125	12	0.049	0.026	0.007	0.042	0.0285	0.078	0.019	0.087
Yes	0.25	12	0.016	0.028	0.008	0.015	-0.013	0.0325	-0.02	0.059
Yes	0.5	12	-0.001	0.013	0.004	-0.0025	-0.009	0.0025	-0.025	0.026
Yes	1	12	-0.009	0.012	0.003	-0.0065	-0.0205	0.001	-0.031	0.006

SD = Standard Deviation
SE = Standard Error
IQR = Interquartile Rang

TABLE II

Pairwise comparison of treatment groups with blue light and NaOCl

Effect	NaOCl	Light	vs.	NaOCl	Light	Rank p-value
NaOCl	0		>	1		<0.0001
NaOCl	0		>	0.5		<0.0001
NaOCl	0		>	0.25		<0.0001
NaOCl	0		>	0.125		0.0006
NaOCl	1		<	0.5		0.0113
NaOCl	1		<	0.25		<0.0001
NaOCl	1		<	0.125		<0.0001
NaOCl	0.5		<	0.25		0.0019
NaOCl	0.5		<	0.125		<0.0001
NaOCl	0.25		<	0.125		<0.0001
Light		0	>		1	<0.0001
NaOCl*Light	0	0	>	0	1	0.0017
NaOCl*Light	0	0	>	1	0	<0.0001
NaOCl*Light	0	0	>	0.5	0	<0.0001
NaOCl*Light	0	0	>	0.25	0	<0.0001
NaOCl*Light	0	0	>	0.125	0	0.0038
NaOCl*Light	0	1	>	1	1	<0.0001
NaOCl*Light	0	1	>	0.5	1	<0.0001
NaOCl*Light	0	1	>	0.25	1	<0.0001
NaOCl*Light	0	1	>	0.125	1	0.0433
NaOCl*Light	1	0	NSD	1	1	0.0614
NaOCl*Light	1	0	<	0.5	0	0.0102
NaOCl*Light	1	0	<	0.25	0	<0.0001
NaOCl*Light	1	0	<	0.125	0	<0.0001
NaOCl*Light	1	1	NSD	0.5	1	0.3037
NaOCl*Light	1	1	<	0.25	1	0.0008
NaOCl*Light	1	1	<	0.125	1	<0.0001
NaOCl*Light	0.5	0	>	0.5	1	0.0007
NaOCl*Light	0.5	0	<	0.25	0	0.0401
NaOCl*Light	0.5	0	<	0.125	0	<0.0001
NaOCl*Light	0.5	1	<	0.25	1	0.0174
NaOCl*Light	0.5	1	<	0.125	1	<0.0001
NaOCl*Light	0.25	0	>	0.25	1	0.0022
NaOCl*Light	0.25	0	<	0.125	0	0.0008
NaOCl*Light	0.25	1	<	0.125	1	<0.0001
NaOCl*Light	0.125	0	>	0.125	1	0.0231

NaOCl*Light = NaOCl treatment groups with or without blue light. A “0” in the light column indicates no light was used whereas a “1” in the light column indicates light was used. The numbers in the NaOCl column represents the concentration of NaOCl used in the experimental group.

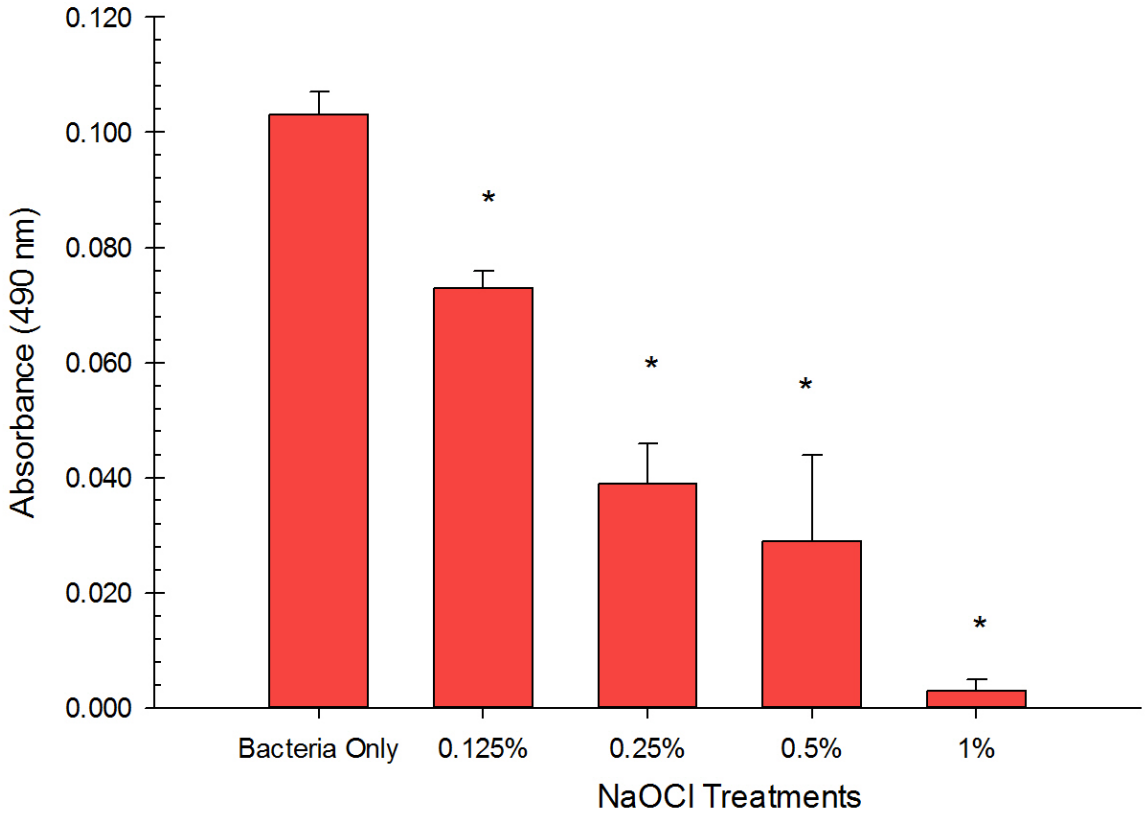

FIGURE 11. Effects of sodium hypochlorite without blue light on reduction of *E. faecalis* biofilm. A* denotes statistically significant difference between bacteria only and NaOCl treatment groups.

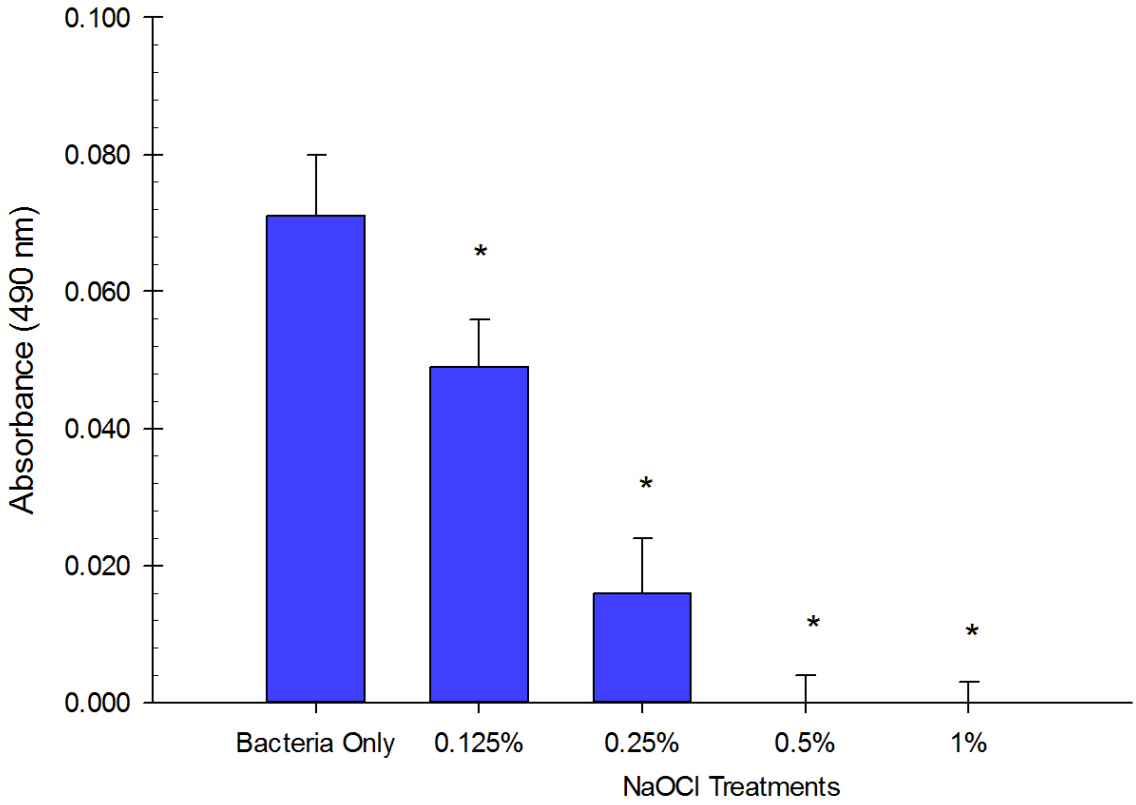

FIGURE 12. Effects of sodium hypochlorite with blue light on reduction of *E. faecalis* biofilm. A * denotes statistically significant difference between bacteria only and NaOCl treatment groups.

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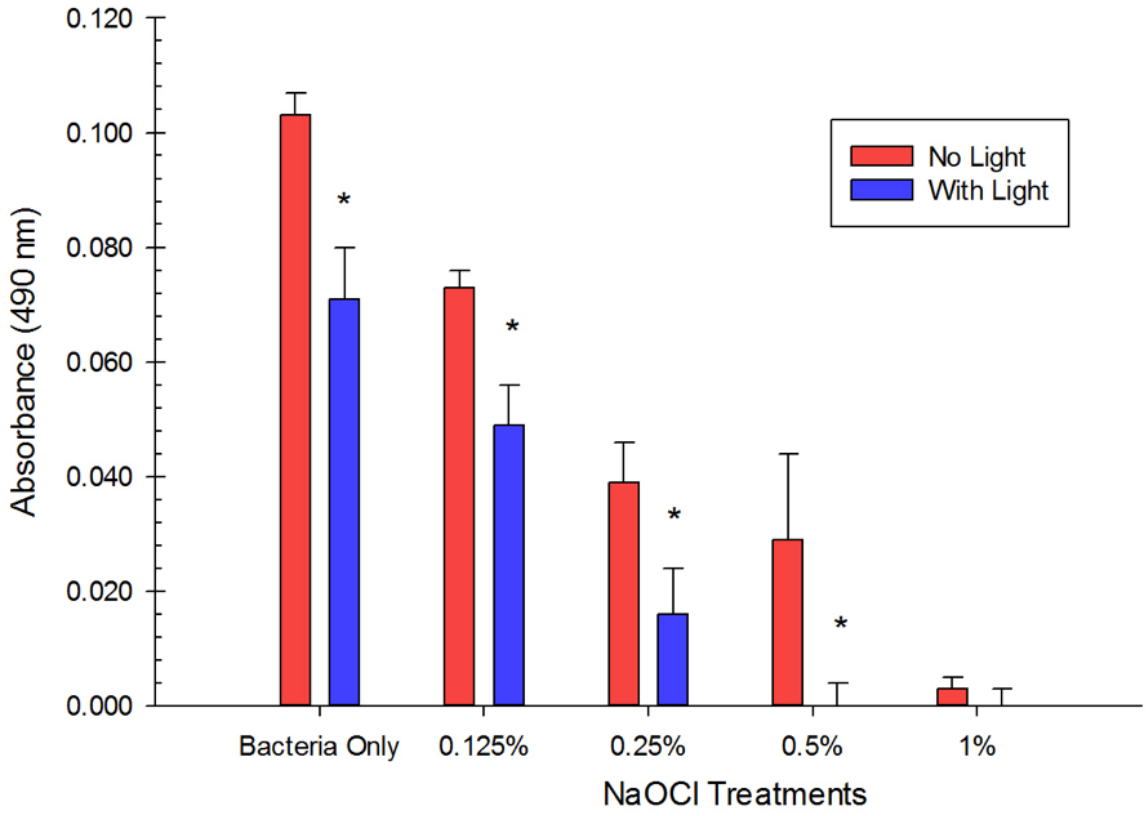

FIGURE 13. Comparison of the effects of sodium hypochlorite with and without blue light on reduction of *E. faecalis* biofilm, A * denotes statistically significant difference between treatment group with light vs corresponding treatment group without light.

DISCUSSION

Based on our results, blue light sourced from a QLF unit at a wavelength of 380 nm to 440 nm, when used in conjunction with NaOCl, will reduce the adherence of *E. faecalis* biofilm. Previous studies have used a laser diode light source at 635 nm to 665 nm (141,142,143) with similar success in reducing *E. faecalis* biofilm. Further studies will be needed to evaluate the efficacy of bacterial reduction at various light sources and wavelengths.

This study used a monospecies biofilm of *E. faecalis* which was incubated for 72 hours. It has been shown that endodontic infections are more polymicrobial in nature (139). Polymicrobial biofilms have a larger genetic diversity, which makes them more tolerant to environmental stresses (144). In addition, persistent endodontic infections arise from a long standing invasion of bacteria capable of forming a mature biofilm. As oral biofilms mature and thicken, they can better establish on a substrate, and in the case of a polymicrobial biofilm community, anaerobic bacteria can reside deeper, which would make them less susceptible to eradication. As a result, follow-up studies should assess the resilience of a mature, polymicrobial biofilm to blue light. Previous studies have used a time period of 3 weeks as a good indicator of an established, mature biofilm (140).

Similar studies involving photodynamic light therapy employ a five- minute treatment time (20). Other studies have also used an exposure time of 120 secs and 240 secs (142,143). It would be of clinical benefit to providers if the treatment could be assessed to investigate if a shorter exposure time would have similar results in reducing biofilm. This would allow for greater operator efficiency and a shorter chair time for the patient.

The blue light delivery system has yet to be investigated in an *in-vivo* study with intact teeth. It is imperative that blue light be introduced down the length of the canal up until full working length for this approach to be effective. Typically, in regenerative procedures, the root apex is immature and has an open apex, which would allow for easier accessibility to the apical third of the root, which is of utmost importance for disinfection when dealing with endodontic infections. On the other hand, if PDT is to be used for conventional root canal treatment, introduction of blue light may prove to be more challenging due to the curvatures and narrow constrictions towards the apex of mature teeth. Previous studies have used fiber optic cables for laser therapy and also for intracanal visualization (145). More studies are required to assess similar delivery methods to determine if they are compatible with PDT using blue light.

Most studies involving the use of PDT have involved the use of an exogenous photosensitizer. Due to the previous research that was done at IUSD (20), the present study used an endogenous photosensitizer that showed promising results. It would be of interest to investigate the difference in biofilm reduction if an exogenous photosensitizer was to be used versus relying on endogenous photosensitizers within the cells.

SUMMARY AND CONCLUSIONS

The conclusions of this study suggest that application of blue light at 380 nm to 440 nm without the use of an exogenous photosensitizer, and used with low concentrations of NaOCl, can be used synergistically to reduce the adherence of *E. faecalis* biofilm. This may allow for increased disinfection of the canal space and can be a great adjunct for regenerative procedures to limit cytotoxicity to apical stem cells from higher concentrations of NaOCl.

Therefore, we reject the null hypothesis and accept the alternative hypothesis that blue light when used in conjunction with NaOCl will reduce the adherence of *E. faecalis* biofilm.

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ABSTRACT

REDUCTION OF *ENTEROCOCCUS FAECALIS* BIOFILM BY BLUE LIGHT AND SODIUM HYPOCHLORITE

by

Daryl A. Kwan

Indiana University School of Dentistry
Indianapolis, Indiana

Introduction: Microbial biofilms have been shown to be a cause of persistent endodontic infections. It is more resistant than planktonic bacteria to host immune defenses and antimicrobials. Studies indicate that photodynamic light therapy (PDT), which involves using light at specific wavelengths, has a potent antibacterial effect on bacterial biofilm. PDT is an antimicrobial strategy that involves the use of a nontoxic photosensitizer (PS) along with a light source. The excited PS reacts with molecular oxygen to produce highly reactive oxygen species, which induce injury or death to microorganisms. PSs have a high degree of selectivity for inhibiting microorganisms without negatively affecting host mammalian cells. PDT has been suggested as an adjuvant to conventional endodontic treatment. Studies at IUSD have shown that blue light at 380 nm to 440 nm has the ability to inactivate *Streptococcus mutans* biofilm without any exogenous PS.

Objective: The objective of this study was to determine the effectiveness of blue light at 380 nm to 440 nm to reduce adherence of *Enterococcus faecalis* biofilm after NaOCl irrigation at various concentrations.

Materials and Methods: *E. faecalis* biofilm was established for 72 hours in 96-well flat-bottom microtiter plates using Tryptic Soy Broth supplemented with 1.0-percent sucrose (TSBS). Biofilm was irradiated with blue light for 5 minutes before exposure to various concentrations of NaOCl for 30 seconds. A crystal violet biofilm assay was used to determine relative density of the biofilm. Data were analyzed with two-way ANOVA and Sidak-adjusted multiple comparisons using a 5.0-percent significance level.

Null Hypothesis: Blue light and NaOCl will not have an effect against *E. faecalis* biofilm adherence.

Results: Overall, there was a significant effect ($p < 0.05$) for NaOCl and a significant effect for blue light. The effects of the combination of NaOCl and blue light were also significant.

Conclusion: We reject the null hypothesis and accept the alternative hypothesis that blue light when used in conjunction with NaOCl will reduce adherence of *E. faecalis* biofilm.

CURRICULUM VITAE

Daryl Anthony Kwan

December 2006	BA, Biology, and BS, Management Case Western Reserve University Cleveland, Ohio
May 2011	DDS, University of Michigan School of Dentistry Ann Arbor, Michigan
July 2011 to June 2012	General Dentistry Atacosa Health Center Pleasanton, Texas
June 2012 to May 2015	General Dentistry Lee Dental Centers San Antonio, Texas
June 2017	MSD, Oral and Maxillofacial Pathology Certificate in Endodontics Indiana University School of Dentistry Indianapolis, Indiana

Professional Organizations

American Association of Endodontists
Texas Dental Association
American Dental Association
San Antonio District Dental Society